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Report

Adaptive Sugar Provisioning Controls Survival of *C. elegans* Embryos in Adverse Environments

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Summary

The ability to adapt to changing environmental conditions is essential to the fitness of organisms. In some cases, adaptation of the parent alters the offspring's phenotype [1–10]. Such parental effects are adaptive for the offspring if the future environment is similar to the current one but can be maladaptive otherwise [11]. One mechanism by which adaptation occurs is altered provisioning of embryos by the parent [12–16]. Here we show that exposing adult *Caenorhabditis elegans* to hyperosmotic conditions protects their offspring from these conditions but causes sensitivity to anoxia exposure. We show that this alteration of survival is correlated with changes in the sugar content of adults and embryos. In addition, mutations in gene products that alter sugar homeostasis also alter the ability of embryos to survive in hyperosmotic and anoxic conditions and engage in the adaptive parental effect. Our results indicate that there is a physiological trade-off between the presence of glycerol, which protects animals from hyperosmotic conditions, and glycogen, which is consumed during anoxia. These two metabolites play an essential role in the survival of worms in these adverse environments, and the adaptive parental effect we describe is mediated by the provisioning of these metabolites to the embryo.

Results

C. elegans embryos can survive a 24 hr bout of anoxia at 20°C–23°C with 95% or greater viability (Table 1) [17, 18]. We performed experiments to determine whether survival could be altered by changing the parental environment. We found that exposing wild-type (N2) L4 larvae to 300 mM sodium chloride for 24 hr as they progressed to adulthood (hyperosmotic preconditioning [OPC]) reduced survival of their embryos in anoxia to a mean of 42% (Table 1; $p < 0.001$). However, embryos from OPC mothers hatched in normoxia and were resistant to 500 mM sodium chloride, a concentration that killed embryos that did not have parental OPC (Table 1; $p = 0.01$). This effect persisted for at least 4 hr after the adults were removed from the high-salt environment (Table 1; $p < 0.001$). These results demonstrate that the parental environment adapts offspring to their probable future environment in an environment-specific fashion. They also show that the embryos are not generally "sick" as a result of parental exposure to hyperosmotic conditions but are actually adapted to the salt in a way that makes them more sensitive to anoxia.

We next tested *daf-2(e1370)* (GI175410) adult nematodes, which carry a hypomorphic allele of the *C. elegans* insulin/insulin-like growth factor (IGF) receptor, for their ability to engage in OPC and found that *e1370* animals were unable to adapt their embryos to survive in hyperosmotic conditions. No embryos from OPC *e1370* mothers hatched on 500 mM salt, whereas ~30% of wild-type embryos hatched in the same conditions (Table 1). These results suggest that the worm insulin receptor plays a role in regulating the adaptive parental effect. To further investigate this, we analyzed a downstream target of *daf-2* signaling, the FOXO transcription factor *daf-16*. We determined that the deficit in OPC found in *e1370* animals was hypostatic to the *daf-16* reference allele *m26*. *daf-16(m26)* mutant embryos performed very similarly to wild-type, whereas *daf-16(m26); daf-2(e1370)* double mutants performed better than wild-type (Table 1; $p < 0.001$ for improvement of *daf-16; daf-2* over N2).

Given the known importance of glycerol and trehalose to osmotic resistance in the nematode [19–22] and glycogen to the survival of a wide variety of animals in anoxia [23, 24], we tested how OPC alters sugar homeostasis in *C. elegans*. We chose to investigate a broad range of sugars via a gas chromatography-mass spectrometry (GC/MS) technique [25, 26]. In wild-type embryos, glycogen levels fell to approximately 20% of their initial levels after 24 hr exposure to anoxia, indicating that fermentative metabolism was continuing in the arrested embryos (Figure 1A; $p < 0.01$). This is consistent with previous studies in mixed-stage animals showing a similar reduction in glycogen stores over a 24 hr period of anoxia [27]. Next, we treated wild-type and *e1370* adults with OPC and looked for alterations in sugar homeostasis in their embryos via the GC/MS technique. Embryos from OPC adults had ten times the glycerol content, nearly twice the trehalose, and only one-third of the glycogen content of control embryos (Figure 1B; $p < 0.01$ for glycerol and glycogen; $p < 0.05$ for trehalose). Glycerol is a nonfermentable sugar and therefore cannot be used for energy during anoxia. Trehalose has also been implicated in salt resistance in *C. elegans*, possibly as a cytoprotectant [22]. *daf-2(e1370)* embryos from OPC adults, on the other hand, had only a 4-fold increase in glycerol levels, no change in glycogen content, and a decrease in trehalose levels (Figure 1B; $p < 0.01$ for trehalose). The deficiency in either glycerol or trehalose may explain why *e1370* embryos are not adapted to their hyperosmotic environment.

We next examined the larval response to preconditioning by GC/MS. These results largely reiterated the observations made in embryos, with the exception that trehalose levels did not rise in wild-type larvae treated with OPC. Glycerol rose in both wild-type and *e1370* after OPC but only reached half of the wild-type level in the *e1370* adults. Glycogen levels dropped by half in wild-type ($p < 0.01$) but were unchanged in *e1370* (Figure 1C). Glycerol accumulation in high-salt conditions requires the glycerol-3-phosphate dehydrogenase enzymes *gpdh-1* (GI173272) and *gpdh-2* (GI176399) [21]. In order to better understand the relationship between glycogen, glycerol, and

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Table 1. An Adaptive Parental Effect Protects *C. elegans* from Hyperosmotic Stress and Requires *daf-2* in a *daf-16*-Dependent Manner

Strain	Adult Treatment	Embryo Treatment	Survival of Embryos	SD	Number of Trials	n	p Value versus Control
N2	50 mM NaCl	50 mM NaCl	98.6%	1.4%	9	768	
	50 mM NaCl	anoxia 23°C	95.7%	3.2%	15	1382	
N2	300 mM NaCl	50 mM NaCl	95.6%	1.7%	7	505	
	300 mM NaCl	anoxia 23°C	41.6%	21.1%	14	741	<0.001[a]
N2	50 mM NaCl	500 mM NaCl	0.4%	5.2%	18	1005	
	300 mM NaCl	500 mM NaCl	26.2%	22.0%	17	789	0.010[b]
	300 mM NaCl + 4 hr 50 mM NaCl	500 mM NaCl	10.3%	7.9%	7	282	<0.001[b]
daf-2(e1370)	50 mM NaCl	500 mM NaCl	1.1%	2.5%	8	475	
	300 mM NaCl	500 mM NaCl	0.0%	0.0%	6	179	0.491[b]
daf-16(m26); daf-2(e1370)	50 mM NaCl	500 mM NaCl	7.2%	7.4%	5	417	
	300 mM NaCl	500 mM NaCl	65.5%	15.4%	5	316	<0.001[a]
daf-16(m26)	50 mM NaCl	500 mM NaCl	1.1%	3.1%	7	532	
	300 mM NaCl	500 mM NaCl	23.3%	22.1%	7	442	0.001[b]

Adult treatment consisted of growing animals from L4 to adulthood on 50 mM NaCl or 300 mM NaCl. Embryo treatment consisted of exposing embryos to 50 mM NaCl or 500 mM NaCl in normoxia or to anoxia for 24 hr (anoxia 23°C). Survival of embryos is shown as mean percent of embryos hatched 24 hr after embryo treatment. SD = standard deviation of the sample mean; n = number of embryos assayed in all trials.
[a] p value determined by two-tailed t test.
[b] p value determined by Mann-Whitney rank sum test.

trehalose, we exposed *gpdh-1(ok1558); gpdh-2(kb33)* mutant animals to OPC and analyzed their sugar profile. As expected, adults treated in this fashion exhibited only a minor increase in glycerol. However, like wild-type, they still utilized two-thirds of their glycogen, and trehalose abundance increased 2.3-fold over its initial level (see Figure S1 available online).

We next stained animals with iodine in order to further analyze how glycogen contributes to survival in adverse environments. Adult worms stained with iodine revealed major glycogen stores immediately anterior to the posterior bulb of the pharynx, in a region of the tail near the dorsorectal ganglion, and in the most proximal two oocytes of the gonad arm (Figure 2A). Iodine staining largely disappeared when glycogen synthase (*gsy-1* [GI174924]) was knocked down by RNA interference (RNAi) (Figure 2B). The small amount of remaining stain may result from incomplete knockdown or the presence of a small quantity of another substance that stains with iodine. Embryos from adults treated with *gsy-1* RNAi were sensitive to anoxia exposure, further verifying the importance of glycogen to survival of embryos in anoxia (Figure 2C; $p < 0.01$). Iodine staining also disappeared upon anoxia or salt exposure, consistent with the decrease in glycogen that we observed by GC/MS in these situations. *daf-2(e1370)* adults exhibited more glycogen than wild-type by iodine staining (Figure S2C), consistent with our GC/MS results and these animals' known resistance to anoxia [28]. For a more complete description of the appearance and dynamics of glycogen in the worm, see the Supplemental Results.

We wished to extend our understanding of how sugars affect survival in adverse environments by studying mutants with altered sugar homeostasis. First we tested the mutant *osm-7(n1515)* (GI176790). Although the molecular function of OSM-7 remains obscure, mutant animals are constitutively adapted to high salt and are known to accumulate glycerol [20]. We found that *osm-7* mutant animals also had low glycogen content and that their embryos were very sensitive to anoxia exposure (average survival 10%) but resistant to 500 mM sodium chloride (Figures 3A and 3B). *dpy-10(e128)* (GI174106) mutant animals, which have a defect in a cuticle collagen, are also known to be resistant to hyperosmotic conditions and to accumulate glycerol [20]. These mutants were also found to be deficient in glycogen and sensitive to anoxia (Figures 3A and 3B). The phenotypes of these mutants reiterate the relationship between glycerol and glycogen as well as survival in hyperosmotic and anoxic conditions that was previously demonstrated by environmental manipulation in wild-type. Neither of the mutants had increased trehalose relative to wild-type, implying that increased glycerol is sufficient for resistance to high salt in embryos.

We also analyzed strains with defects in glucose metabolism in order to determine how this affects survival in anoxic and hyperosmotic environments. Three mutants involved in gluconeogenesis were identified that were sensitive to anoxia and hyperosmotic conditions (Figure 3A). One strain has a mutated phosphoenolpyruvate carboxykinase enzyme (*PEPCK/W05G11.6* [*ok2098*] [GI175171]). This strain lays embryos that are sensitive to anoxia (average survival 50%; $p < 0.01$), though with high variability. We could not test the adaptation of *PEPCK* mutant embryos to salt because, unlike wild-type, very few *ok2098* L4 animals develop into egg-laying adults when placed on 300 mM NaCl. The other two strains have mutations in the bifunctional isocitrate lyase/malate synthase enzymes (*gei-7(ok531)* [GI178583]; *C08F11.14(ok457)* [GI178328]), key components of the glyoxylate cycle. Each of the individual glyoxylate cycle mutants has a weaker anoxia sensitivity phenotype (50% and 85% survival, respectively; data not shown) than the double-mutant strain (Figure 3A; 35% survival; $p < 0.01$). The embryos from glyoxylate cycle double mutants treated with OPC were not adapted to high salt and fared even worse in anoxia after OPC. No glyoxylate cycle mutant embryos survived in 500 mM salt after OPC, and 1.3% survived in anoxia after OPC, compared to 26% and 42% for wild-type, respectively ($p < 0.05$ for both). Although the exact reason that these mutants are sensitive to both salt and anoxia has not been fully elucidated, the data from these experiments suggest a strong correlation between the ability to store glycogen and survival in both anoxia and high-salt environments.

Discussion

We have provided the first evidence that *C. elegans* adults are capable of preparing their embryos to survive in a specific environment. This adaptive parental effect, induced by

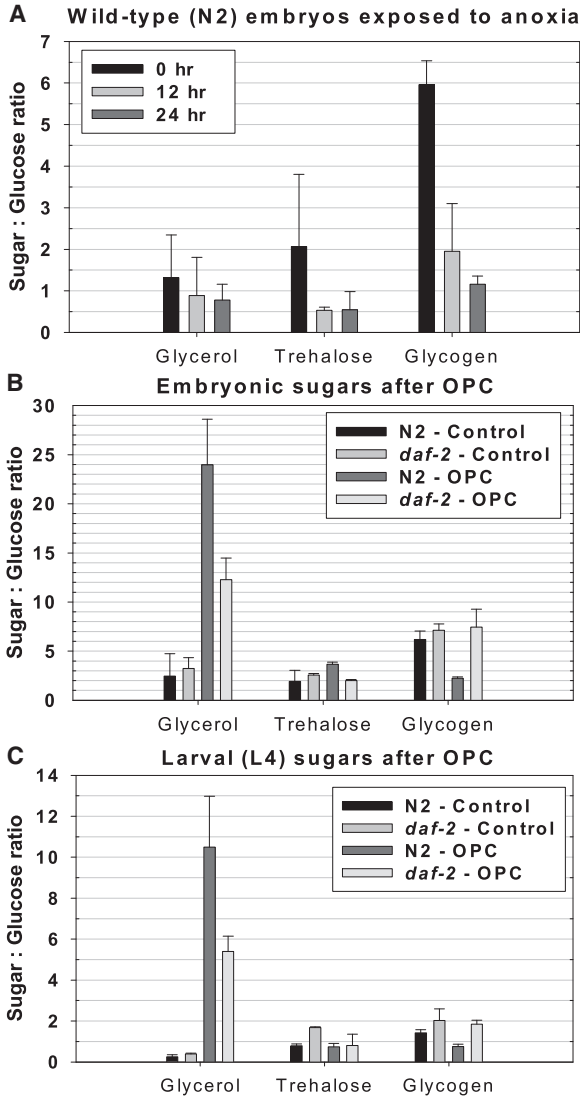


Figure 1. Hyperosmotic Preconditioning-Mediated Adaptive Sugar Provisioning in *C. elegans* Requires *daf-2*

The graphs display the quantity of each sugar relative to the quantity of glucose in the animals, as described in Supplemental Experimental Procedures. Values are presented as the mean of 3–6 replicates, ±SD.

(A) Young embryos were collected and placed into anoxia, with samples taken for sugar analysis at t = 0, 12, and 24 hr. Over the 24 hr incubation, glycogen levels fell to 1/5 of their initial level ($p < 0.01$).

(B) Wild-type (N2) embryos from hyperosmotically preconditioned (OPC) adults contain much more glycerol, almost twice the amount of trehalose, and one-third of the glycogen of control animals ($p < 0.01$ for glycerol and glycogen; $p < 0.05$ for trehalose). *daf-2* embryos only accumulate one-half of the glycerol of wild-type after OPC, have decreased trehalose levels, and exhibit no change in glycogen storage ($p < 0.01$ for trehalose).

(C) Changes in larval sugar levels reiterate the observation in embryos, with the exception that trehalose levels do not increase in wild-type larvae treated with OPC.

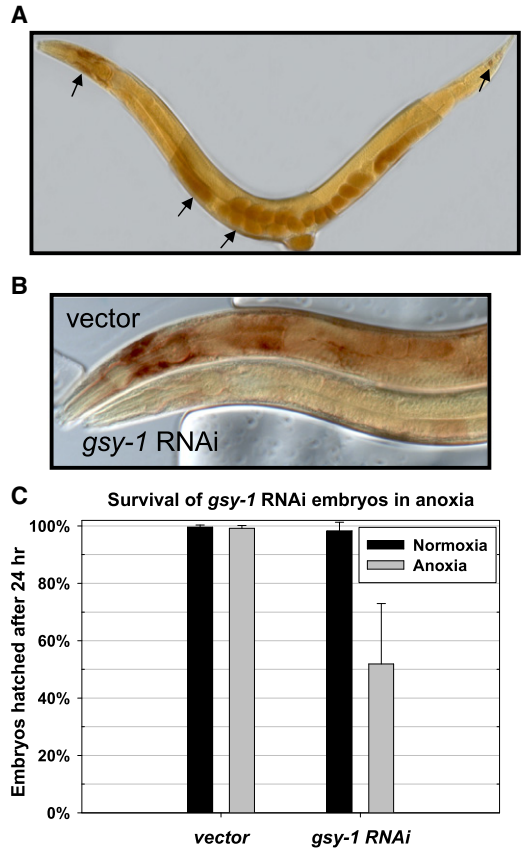


Figure 2. Iodine Staining Reveals Glycogen Storage in *C. elegans*, and Glycogen Synthase Contributes to Survival of Embryos in Anoxia

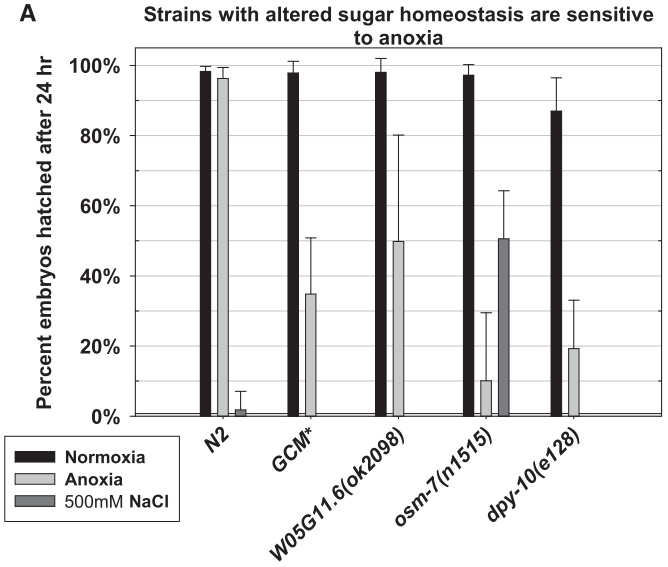
(A) Wild-type (N2) adult animal stained with iodine vapor. Arrows denote the primary sites of glycogen deposition. From left to right, these sites are: anterior to the posterior bulb of the pharynx, the proximal oocytes, embryos in utero, and the tail hypodermis.

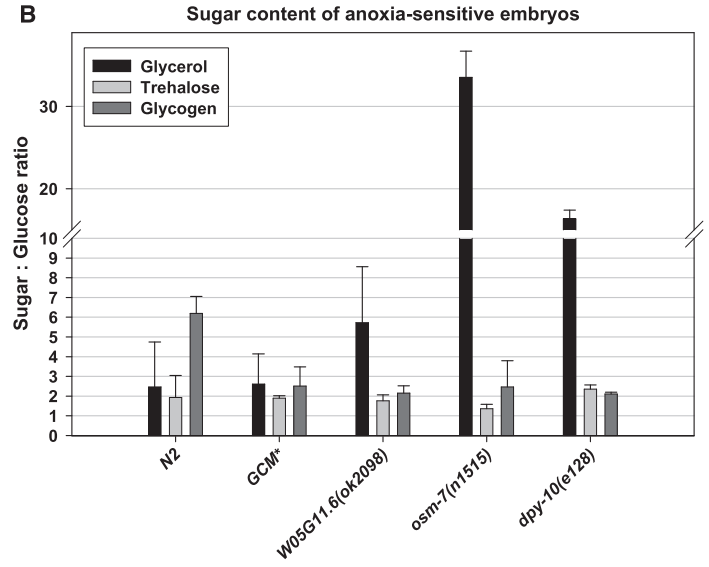
(B) A wild-type (N2) animal fed with empty vector RNA interference (RNAi) control food (top) stained with iodine simultaneously with an N2 animal treated with glycogen synthase (*gsy-1*) RNAi (bottom).

(C) *gsy-1* RNAi renders embryos sensitive to 24 hr anoxia at 23°C. Values are the mean percentage of animals surviving anoxia exposure from 14 trials, ±SD.

hyperosmotic preconditioning, is associated with increased glycerol and decreased glycogen in the adult worm and the same changes in the embryo. We find it particularly interesting that trehalose levels remain constant in the OPC adult but increase in the embryo, because this suggests an alteration in embryonic provisioning that is not merely reflective of parental physiology. Both glycerol and trehalose have previously been shown to be important for survival in hyperosmotic conditions [19, 21, 22].

daf-2(e1370) adults are unable to engage in this adaptive parental effect, whereas *daf-16(m26); daf-2(e1370)* animals perform better than wild-type. The evidence suggests that *daf-2* mutant animals are incapable of correctly provisioning their embryos with the level of glycerol or trehalose required to survive in high-salt environments and that alterations in metabolism responsible for this defect operate through the FOXO transcription factor *daf-16*. The metabolic origin of this defect is difficult to hypothesize a priori, because gene expression analyses and other studies have suggested that *daf-2(e1370)* adults have metabolic activity very different from wild-type [29–35]. The high level of glycerol accumulated by wild-type animals upon OPC is correlated with a decrease in glycogen storage, which did not occur in OPC *e1370* adults. Glycerol accumulation in high salt requires *gpdh-1* and *gpdh-2*; this strongly suggests that the source of the glycerol is the glycolytic intermediate dihydroxyacetone phosphate (DHAP), which may be produced from either gluconeogenesis





* Glyoxylate cycle double mutant (*gei-7*(*ok531*)V; *C08F11.14*(*ok457*)IV)

Figure 3. Mutations that Alter Sugar Homeostasis Affect the Survival of Embryos in Adverse Environments

(A) Graph displaying the mean percent of embryos hatching 24 hr after the treatments described, with error bars representing the standard deviation of the mean (n = number of embryos assayed). N2 embryos survive 24 hr anoxia but die when exposed to 500 mM NaCl ($p < 0.01$). Glyoxylate cycle double mutant (GCM; *gei-7(ok531); C08F11.14(ok457)*) embryos are sensitive to anoxia (n = 277 normoxia; n = 1026 anoxia; $p < 0.01$). *W05G11.6* (*ok2098*) embryos are sensitive to anoxia (n = 150 normoxia; n = 299 anoxia; $p < 0.01$). *osm-7* embryos are sensitive to anoxia (n = 572 normoxia; n = 600 anoxia; $p < 0.01$). *osm-7* embryos are resistant to 500 mM NaCl as compared to wild-type (n = 252 500 mM NaCl; $p < 0.01$). *dpy-10(e128)* animals are sensitive to anoxia (n = 83 normoxia; n = 100 anoxia; $p < 0.01$).

(B) Embryos from each of the strains that have decreased survival in anoxia also have decreased glycogen storage compared to wild-type (N2) embryos ($p < 0.01$ for all comparisons). Both *osm-7* and *dpy-10* embryos accumulate glycerol, as well as being deficient in glycogen. Glycerol accumulation is associated with resistance to hyperosmotic conditions. Values are the mean of three replicates ± SD.

or glycolysis. Triglyceride catabolism, which produces glycerol without glycerol-3-phosphate dehydrogenase, is not likely to be an important source for glycerol upon hyperosmotic exposure. Trehalose was still produced and glycogen was still consumed when we exposed *gpdh-1; gpdh-2* mutants to OPC, so trehalose production and glycogen consumption are most likely independent of glycerol production. Among the possible explanations for the lack of glycerol and trehalose accumulation in *daf-2(e1370)* animals is that these worms have a dampened environmental response and as a result do not adequately shunt glycolytic intermediates into either glycerol or trehalose, thereby avoiding the need to consume their glycogen stores. Another possibility is that either trehalose or glycerol is produced directly from glycogen and that this function is deficient in *daf-2(e1370)* animals, though the quantity of glycerol produced seems to be far in excess of glycogen stores. Metabolic tracer studies will likely be required in order to successfully disentangle these possibilities. Because *daf-2(e1370)* adults are known to be long lived and resistant to a variety of stresses, including hyperosmotic stress and anoxia, we wonder whether the improved stress resistance of the adult may be detrimental to its offspring [22, 28, 36].

In order to learn more about the relationship between sugars and survival in adverse conditions, we investigated several mutants with defects in sugar homeostasis. Based upon these studies and our work in wild-type animals, we suggest that survival in hyperosmotic and anoxic conditions is antithetical for *C. elegans* and that the mechanistic basis for this may be the apparently obligatory trade-off between the abundance of glycerol and glycogen. RNAi of *gsy-1*, which encodes the worm glycogen synthase enzyme, results in low glycogen and sensitivity of embryos to anoxia. Mutants that are known to have increased osmotic resistance and to accumulate glycerol, such as *osm-7(n1515)* and *dpy-10(e128)*, are also sensitive to anoxia and low in glycogen. We hypothesize that many strains that accumulate glycerol as a means of compensating for defects in protein homeostasis or collagen structure will be sensitive to anoxia. Worms with defects in genes required for gluconeogenesis (glyoxylate cycle genes *gei-7* and *C08F11.14* as well as PEPCK/*W05G11.6*) have low glycogen content and are sensitive to both anoxic and hyperosmotic environments, implying that glycogen in particular or functional sugar metabolism in general is required for survival in both situations. These conclusions are supported by our biochemical analysis of carbohydrate changes upon anoxia and hyperosmotic stress in wild-type worms, which shows that glycerol is produced and glycogen consumed as a response to hyperosmotic environments and that glycogen is consumed in anoxia.

Supplemental Data

The Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, and two figures and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)00925-7.

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